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(57) Abstract

A method for amplifying nucleic acid sequences in a template comprising at least one nucleic acid or mixture of nucleic acids wherein each nucleic acid comprises at least one strand. The method comprises the steps of treating the template with at least one oligonucleotide primer having a nucleic acid sequence that is substantially complementary to at least one nucleic acid sequence on the template under conditions such that the primer will bind to a segment that is substantially complementary to the primer, producing primed strands of nucleic acid. The primed strands produce extension strands, wherein the extension strands comprise the primer in combination with a sequence of nucleic acids that is substantially complementary to the template in a 5′ to 3′ direction from the primer. The extension strands are further treated with the primer producing amplification strands which are treated with primer. The amplification strands are repeatedly treated with primer to produce large numbers of amplification strands whereby the nucleic acid sequences of the amplification strands are substantially similar to nucleic acid sequences in the original template.

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+ DESIGNATIONS OF "SU"

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DNA AMPLIFICATION FINGERPRINTING

The present invention relates to a process for amplifying nucleic acid sequences present in a sample of nucleic acid and the detection of those nucleic acid sequences. More specifically, the present invention relates to a process for producing characteristic but unspecified sequences from a given sequence of nucleic acids in amounts which are large compared to the amount initially present. The process of the invention utilizes a repetitive reaction to accomplish the amplification of the desired nucleic acid sequence.

DNA profiling, also known as DNA "fingerprinting", has become a significant technology for the study of the human genome, linkage analysis, genetic predisposition to disease, parentage testing and genetic variation, and a powerful identification tool in forensic medicine and epidemiology. In an early technique of DNA fingerprinting restriction enzymes are used to cut sample DNA into millions of pieces, including fragments that contained repeated segments. The fragments produced are then separated by gel electrophoresis. Using this technique, the concentrations of the fragments are so low that they must be identified with a cloned radioactive probe. Since the probe is designed to identify only one particular sequence of nucleic acid, only a few of the millions of segments are identified. When the autoradiograph is developed, the resulting bands produce restriction fragment length polymorphisms (RFLP). Where there is a difference between the length of the fragments produced by the restriction enzyme those fragments are considered to be polymorphous. The polymorphous and non-polymorphous fragments are used to determine whether or not two samples belong to the same organism. Although this technique is widely used, the technology requires considerable DNA manipulation and is dependent upon the availability of cloned DNA probes to identify differences between fingerprints.

The polymerase chain reaction (PCR) is a key

procedure for molecular biology research which has also been used for DNA fingerprinting. The PCR technique is an in vitro method in which a genomic or cloned target sequence is enzymatically amplified as directed by a pair of highly specific oligonucleotide primers. The amplification results from a repetitive reaction which produces large amounts of target sequence DNA when compared to the amount initially present in the DNA sample. PCR relies on stringent DNA hybridization and enzymatic reaction conditions as well as absolute primer specificity. In addition the PCR reaction requires two unique primers, each typically over twenty base pairs in length, for the amplification of a single target sequence.

It is an object of the present invention to provide a method for the amplification of unspecified but characteristic sequences of DNA. It is an object of the invention to provide this amplification without the DNA manipulation required by the RFLP technique or the specificity of the PCR technique. It is another object of the invention to provide a relatively simple method for profiling the DNA of an organism.

The present invention provides a method for amplifying nucleic acid sequences in a template comprising at least one nucleic acid or mixture of nucleic acids, wherein each nucleic acid comprises at least one strand having one 3' end and one 5' end. The method comprises the steps of first treating the template with at least one oligonucleotide primer having a nucleic acid sequence that is substantially complementary to at least one nucleic acid sequence on the template under conditions such that the primer will bind to a segment of the template that is substantially complementary to the primer, producing primed strands of nucleic acid. Next, the primed strands produce extension strands of nucleic acid wherein the extension strands comprise the primer in combination with a sequence of nucleic acids that is substantially complementary to the template in a 5' to 3' direction from the primer. The extension strands are then treated with the primer pro-

ducing amplification strands of nucleic acid. The amplification strands are also treated with the primer to produce further amplification strands. The treatment of the amplification strands with primer is repeated a number of times to produce large numbers of amplification strands. The nucleic acid sequences of the amplification strands are substantially similar to nucleic acid sequences in the original template. Therefore, the amplified segments of nucleic acid are representative of the nucleic acid sample.

The method of the present invention is readily distinguishable from both the RFLP and PCR techniques. RFLP produces millions of bands using restriction nucleases, but these bands are at low concentrations. Therefore, millions of bands are available for nucleic acid profiling, but particular probes must be used to visualize any of the millions of bands available. Under practical circumstances, only a few of the bands are visualized. The specificity of PCR primers gives a few bands at amplified concentrations. Therefore all of the bands produced may be easily visualized, but only a few bands are available for profiling. In contrast to RFLP and PCR, the method of the present invention produces many bands (tens or hundreds of bands) at amplified concentrations which are easily visualized. Therefore, the method of the present invention retains the advantages of the previous techniques without the shortcomings of either technique.

In brief, the present invention provides a method for amplifying nucleic acid sequences in a template, said template comprising at least one nucleic acid or mixture of nucleic acids, wherein each nucleic acid consists of at least one strand having one 3' end and one 5' end, said method comprising, in order, the steps of:

(a) treating said template with at least one oligonucleotide primer having a nucleic acid sequence that is substantially complementary to at least one nucleic acid sequence on said template under conditions such that said primer will bind to a segment of said

template that is substantially complementary to said primer, thereby producing primed strands of nucleic acid;

(b) producing extension strands of nucleic acid, wherein said extension strands comprise said primer in combination with a sequence of nucleic acids that is substantially complementary to said template in a 5' to 3' direction from said primer;

(c) treating said extension strands with said primer, thereby producing primed extension strands of nucleic acid;

(d) producing amplification strands of nucleic acid, wherein said amplification strands comprise said primer in combination with a sequence of nucleic acids that is substantially complementary to said extension strands in a 5' to 3' direction from said primer;

(e) treating said amplification strands with said primer, thereby producing primed amplification strands of nucleic acid;

(f) producing further of said amplification strands, wherein said further amplification strands comprise said primer in combination with a sequence of nucleic acids that is substantially complementary to said first amplification strands in a 5' to 3' direction from said primer; and

(g) repeating steps (e) and (f), in order, a plurality of times, thereby producing large numbers of said amplification strands, whereby the nucleic acid sequences of said amplification strands are substantially similar to nucleic acid sequences in said nucleic acid or mixture of nucleic acids.

For nucleic acid profiling, the presence of an abundance of bands implies, in general, a greater ability

to discriminate between different samples than the presence of a few bands. For example, the presence of 33 independently variable bands in a nucleic acid profile will be able to distinguish between more than eight billion individuals, where the presence of only 10 independently variable bands would distinguish between only about one in a thousand. Depending on the identity of the primer chosen and the type of nucleic acid template, the method of the present invention will typically produce greater than about 50 bands, and will often produce greater than about 100 visualized bands. Many of the bands will vary independently from individual to individual. Further, the use of additional different primers, either together or separately, will produce other independently variable bands. Therefore, the method of the present invention allows for the identification of nucleic acid materials of a distinct individual from an entire population of individuals.

The process may be carried out in a single vessel for each sample of nucleic acid. The sample of nucleic acid and resulting amplified strands of nucleic acid are repeatedly separated by heating. Upon cooling, primer sequences attach to complementary nucleic acid sequences in solution. A polymerase then allows the formation of a complementary strand of nucleic acid synthesized in the 5' to 3' direction from the primer along the nucleic acid strand. After a short period of time, the temperature of the solution is again raised in order to denature the nucleic acid strands forms. This repetitive cycle provides large numbers of nucleic acid sequences which are the same as segments in the original sample nucleic acid.

The resulting nucleic acid strands are subjected to gel electrophoresis for separation by size. The distribution of amplified segments along the length of the gel represents a unique picture of the nucleic acid sample.

The primers used in the invention range in size from greater than five base pairs. Primers less than five base pairs in length generally do not produce recognizable fragments for nucleic acid fingerprinting. There is no

specific required sequence for any particular primer used in this technique. Any sequence of nucleotides may be used as a primer so long as the same sequence is used when comparing different DNA samples. The fingerprint produced by a particular primer sequence will be unique to that sequence and will be repeatable when using this method with the same sample at two different times.

The present invention has the added advantage of not requiring narrowly specific reaction conditions. The present invention will produce repeatable and distinguishable fingerprints under a wide range of temperature conditions. The reaction mixture may be heated to as high as 98°C and allowed to cool to as low as room temperature. A typical reaction involves 30-50 cycles of 1 sec. at 95°C, 10 sec. at 30°C and 3 min. at 72°C.

In addition to fragments prepared according to the present invention, the amplified fragments may be further treated using restriction enzymes to produce subfragments which may be used for further identification of particular nucleic acid sequences.

The present invention may be better understood by reference to the following detailed description of the invention when considered in conjunction with the drawings in which:

FIGURE 1 is a diagram depicting the method of the present invention;

FIGURE 2 depicts an electrophoresis gel which includes nucleic acid profiles from several different species using the primer with a sequence of 5'-CGCGGCCA-3' demonstrating the profiling capability of the present invention; and

FIGURE 3 depicts an electrophoresis gel which includes DNA profiles of the soybean Glycine max cv. Bragg using primers of different lengths and sequences.

Referring now to FIGURE 1, the method of the present invention is used with an original sample of DNA isolated by any one of a number of methods known in the art (e.g.,R.H. Chesney, J. R. Scott and D. J. Vapnek, J.

Mol. Biol., vol. 130, pp. 161-173 [1979]). The original template of nucleic acid 10 is denatured to form two complementary strands 12 and 14. The complementary strands 12 and 14 are treated with a primer 16 comprising generally between 5 and 25 nucleotides. Primers of less than 5 nucleotides in length generally do not produce recognizable fragments for profiling. Primers of greater than about 25 nucleotides generally produce an insufficient number of amplified segments unless a particular nucleotide sequence is utilized. The sequence of the nucleotides is known but it is not important to the present invention that the sequence be complementary to a particular known segment on the template nucleic acid 10. The primer attaches to the complementary strands 12 and 14 at sites which are complementary to the nucleotides comprising the primer 16, forming primed complementary strands. The primed complementary strands are treated with a nucleic acid polymerase thereby generating extension strands of nucleic acid 18a-18i. The extension strands 18a-18i comprise a primer segment 16 in combination with a sequence 20a-20i of nucleic acids that is substantially complementary to the section of the complementary strands of nucleic acid 12 and 14. Each extension strand 18a-18i extends along the complementary strand 12 or 14 from the 5' end toward the 3' end of the extension strand 18a-18i. The extension strands 18a-18i have a length, as a maximum, of from its own primer 16 to a point 22a-22g just prior to the next primer 16 in the 3' direction. The DNA is then denatured and the extension strands 18a-18i are treated with additional primer 16 forming primed extension strands where the primer 16 is attached to the extension strands 18a-18i at sites which are complementary to the primers 16. Treatment of the primed extension strands with a nucleic acid polymerase produces amplification strands 24a-24g of nucleic acid, each strand 24a-24g comprising a primer 16 in combination with a sequence 26a-26g of nucleic acids that is substantially complementary to the section of the extension strands 18a-18i. The amplification strands 24a-24g are

generated by the polymerase in the direction of the 3' end of the amplification strands 24a-24g and will be generated to the 5' end 28a-28e of any segment of an extension strand 18a-18i. For example, amplification strand 24a is generated to the 5' end 28a of extension strand 18c. The nucleic acid is then denatured producing free amplification strands 24a-24g. The amplification strands 24a-24g are treated with primer 16 forming primed amplification strands. Further treatment of the primed amplification strands with a nucleic acid polymerase produces additional amplification strands 30a-30d, each strand 30a-30d comprising a primer 16 in combination with a sequence 32a-32d of nucleic acids that is substantially complementary to the amplification strand 24a-24d. The amplification strand 24a is the complement of the amplification strand 30a, the amplification strand 24b is the complement of the amplification strand 30b and so forth. In this way, particular segments of nucleic acid are made available for further reproduction. The steps of denaturation, treating with primer 16 and treating with polymerase are repeated. Each further treatment will double the number of amplification strands 24a-24d and 30a-30d leading to a logarithmic increase in the number of amplification strands available for analysis. In the actual practice of the present invention, the repetitive reaction of denaturation, followed by primer treatment, followed by polymerase treatment is accomplished by combining the original nucleic acid sample with excess primer and nucleotides and the cycling of the reaction vessel through changes in temperature.

Upon completion of the amplification of the nucleic acid fragments, the fragments are separated by gel electrophoresis and visualized, as with a silver stain. FIGURE 1 illustrates the production of two different strands which are amplified by the method of the present invention. However, in operation, a large number of strands will be amplified, separated and visualized as is illustrated in FIGURES 2 and 3.

The primers are greater than five nucleotides in

length. Preferentially, the primers are about ten nucleotides in length. Primers with fewer than five nucleotides do not produce amplified fragments necessary for DNA profiling. Primers much larger than about 25 nucleotides produce insufficient amplified segments unless a particular nucleotide sequence is utilized. Although a particular nucleotide sequence may be used according to the method of the present invention, it is not necessary to use a particular nucleotide sequence for the primers of the present invention. It is necessary, however, to use the same primer when comparing different samples for polymorphisms.

In order to provide a further understanding of the invention, the following examples are given primarily for the purposes of illustrating certain more specific details thereof.

EXAMPLE

Nucleic acid samples were obtained according to established procedures (e.g., R. H. Chesney, J. R. Scott and D. J. Vapnek, J. Mol. Biol., vol. 130, pp. 161-173 [1979], S. L. Dellaporta, J. Wood and J. B. Hicks, Plant Mol. Biol. Reporter, vol. 1, pp. 19-21 [1983] and A. J. Jeffreys, V. Wilson and S. L. Thein, Nature, vol. 314, pp. 67-73 [1985]). Amplification was done in a total volume of 100 µl with 10-20 pg of sample nucleic acid, 1 µg of primer (an oligonucleotide of 5-9 base pairs) and 2.5 units of a nucleic acid polymerase (AmpliTaq DNA polymerase from Perkin-Elmer/Cetus) in a reaction buffer comprised of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$ and 0.01% (w/v) gelatin (from Sigma). Deoxyribonucleoside triphosphates (dATP, dCTP, dGTC, dTTP) are also added to the reaction buffer at a concentration of 200 µM each. The reaction mix was overlayed with 2 drops of mineral oil, incubated for 5 minutes at 95°C and amplified in a thermocycler (from Ericomp) connected to a refrigerated water bath for 30-50 cycles consisting of 1 sec at 95°C, 10 sec at 30°C and 3 minutes at 72°C. The heating and cooling rates of the thermocycler were 23°/min. and 14°/min. respectively.

Sample temperature was continuously monitored with a thermal probe. Amplification fragments were separated by polyacrylamide gel electrophoresis using a Mini-Protean II (from Bio-Rad) with 0.45 mm thick slab gels of 5% acrylamide and 7M urea (from Bio-Rad) with a ratio of acrylamide to piperazine diacrylamide (from Bio-Rad) 20:1. Gels and running buffer were prepared from TBE buffer comprising 100 mM Tris-HCl, 83 mM boric acid and 1 mM Na_2EDTA at pH = 8.0. Electrophoresis was conducted at 70V until the dye front (xylene cyanol FF) reached the bottom of the gel. The bands of nucleic acid fragments were visualized by silver staining according to D. Goldman and C. R. Merril, Electrophoresis, vol. 3, pp. 24-26 (1982).

FIGURE 2 shows the DNA profiles of several different species using a single primer with a sequence of 5'-CGCGGCCA-3' wherein the nucleic acids of different organisms treated with the same primer produce varying patterns. Each organism produces a different series of bands after the amplification process of this invention. For example, experiment b shows the pattern of the nucleic acid of a caucasian human which is distinctly different from all other patterns shown in FIGURE 2. However, comparison of experiments d, e and f show that closely related species may also be identified. Experiments d-f show the DNA profiles of soybeans. Experiments d and e are of the species Glycine max and experiment f shows the profile of the soybean Glycine soja. It is clear, from the number of polymorphous bands, that G. max and G. soja are related. However, it is also clear that the nucleic acids of experiments d and e are more closely related to each other than they are to the nucleic acid of experiment f. Experiment d used the cultivar Bragg where experiment f used the cultivar Peking. These two cultivars show many polymorphous bands but there are also nonpolymorphous bands as well. In this way, it is possible to distinguish two cultivars of the same species.

FIGURE 2 also shows that the technique of the present invention is applicable to a wide range of

organisms. Experiment b uses DNA from mammalian tissue where experiments c-h utilize plant tissue and experiment i uses DNA from a phage. Table I shows the specifics of experiments a-i.

TABLE I

Experiment	Organism	Comment
a	Hae III digest of phage X174	Size Standard
b	caucasian human	--
c	Cornus Florida cv. Barton	Dogwood
d	Glycine max cv Bragg	Soybean
e	Glycine max cv. Peking	Soybean
f	Glycine soja PI 468.397	Soybean
g	bermudagrass	--
h	centipedegrass	--
i	lambda phage c1857sam7	--
j	Bst NI digest of plasmid pBR322	Size standard

FIGURE 3 shows the profiles of a single species of soybean, G. max cv. Bragg, using primers of different lengths and sequences. Experiments k-n show the repeatability of the technique when applied to different samples from the same cultivar. All four samples were treated with the primer CGCGGCCA and there is no visible difference between the four profiles. In addition, experiments o-q show the profiles with primers different from that used in experiments k-n. Experiments r and s show that tetrameric primers do not produce the bands necessary for nucleic acid profiling according to the present invention. The details of the profiles shown in FIGURE 3 are given in Table II.

TABLE II

Experiment	Primer (5'-3')	Comment
k	CGCGGCCA	8-mer
l	CGCGGCCA	8-mer
m	CGCGGCCA	8-mer
n	CGCGGCCA	8-mer
o	CGGCGGCGG	9-mer
p	AATGCAGC	8-mer
q	CCTGT	5-mer
r	ACGT	4-mer
s	GCGC	4-mer
t	(Bst NI digest of plasmid pBR322)	SIZE standard

From the above it may be seen that the present invention provides a method for the amplification of unspecified but repeatable sequences of DNA. Further, the invention provides this amplification without the DNA manipulation required by the RFLP technique or the specificity of the PCR technique. Further, the invention provides a relatively simple method for profiling the DNA of an organism. In addition, the invention may be applied to a wide range of organisms using a wide range of primers.

Various features of the invention which are believed to be new are set forth in the appended claims.

WHAT IS CLAIMED IS:

Claim 1. A method for amplifying nucleic acid sequences in a template, said template comprising at least one nucleic acid or mixture of nucleic acids, wherein each nucleic acid consists of at least one strand having one 3' end and one 5' end, said method comprising, in order, the steps of:

(a) treating said template with at least one oligonucleotide primer having a nucleic acid sequence that is substantially complementary to at least one nucleic acid sequence on said template under conditions such that said primer will bind to a segment of said template that is substantially complementary to said primer, thereby producing primed strands of nucleic acid;

(b) producing extension strands of nucleic acid, wherein said extension strands comprise said primer in combination with a sequence of nucleic acids that is substantially complementary to said template in a 5' to 3' direction from said primer;

(c) treating said extension strands with said primer, thereby producing primed extension strands of nucleic acid;

(d) producing amplification strands of nucleic acid, wherein said amplification strands comprise said primer in combination with a sequence of nucleic acids that is substantially complementary to said extension strands in a 5' to 3' direction from said primer;

(e) treating said amplification strands with said primer, thereby producing primed amplification strands of nucleic acid;

(f) producing further of said amplification strands, wherein said further amplification strands comprise said primer in combination

with a sequence of nucleic acids that is substantially complementary to said first amplification strands in a 5' to 3' direction from said primer; and

(g) repeating steps (e) and (f), in order, a plurality of times, thereby producing large numbers of said amplification strands, whereby the nucleic acid sequences of said amplification strands are substantially similar to nucleic acid sequences in said nucleic acid or mixture of nucleic acids.

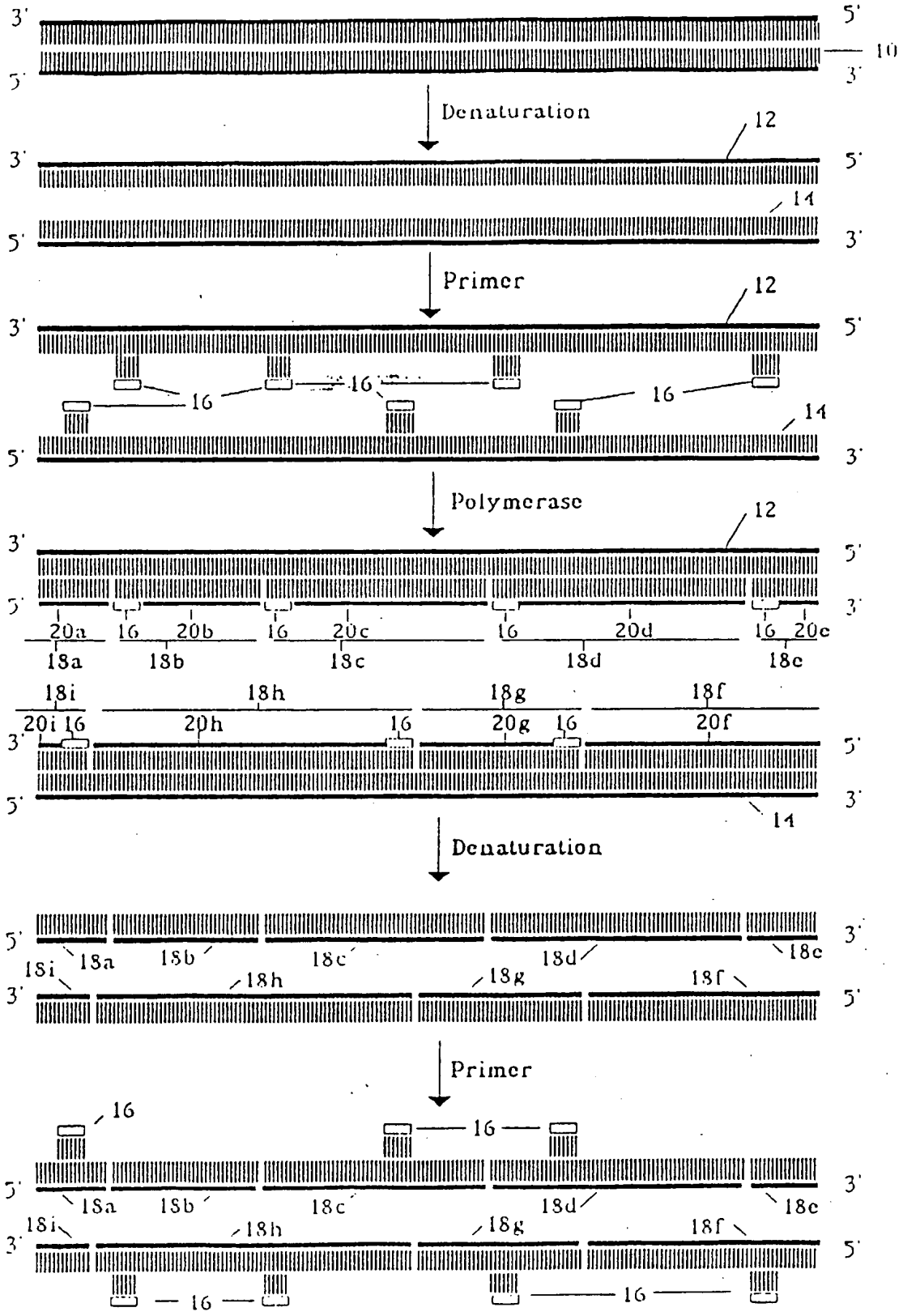
Claim 2. The method of Claim 1 wherein the nucleic acid sequence of said primer is not known to be substantially complementary to any nucleic acid sequence of said template.

Claim 3. The method of Claim 1 wherein said primer comprises an oligonucleotide of at least about 5 nucleotides but less than about 25 nucleotides in length.

Claim 4. The method of Claim 3 wherein said primer is between about 5 and about 10 nucleotides in length.

Claim 5. The method of Claim 1 wherein said oligonucleotide primers comprise a plurality of different nucleic acid sequences.

Claim 6. The method of Claim 1 and including the step of treating said amplification strands with restriction enzymes producing restricted amplification strands of nucleic acid.


3'
5'
10
Denaturation
12
14
Primer
16
Polymerase
20a
20b
20c
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18a
18b
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18i
18h
18g
18f
20i
20h
20g
20f
Denaturation
Primer

Fig. 1

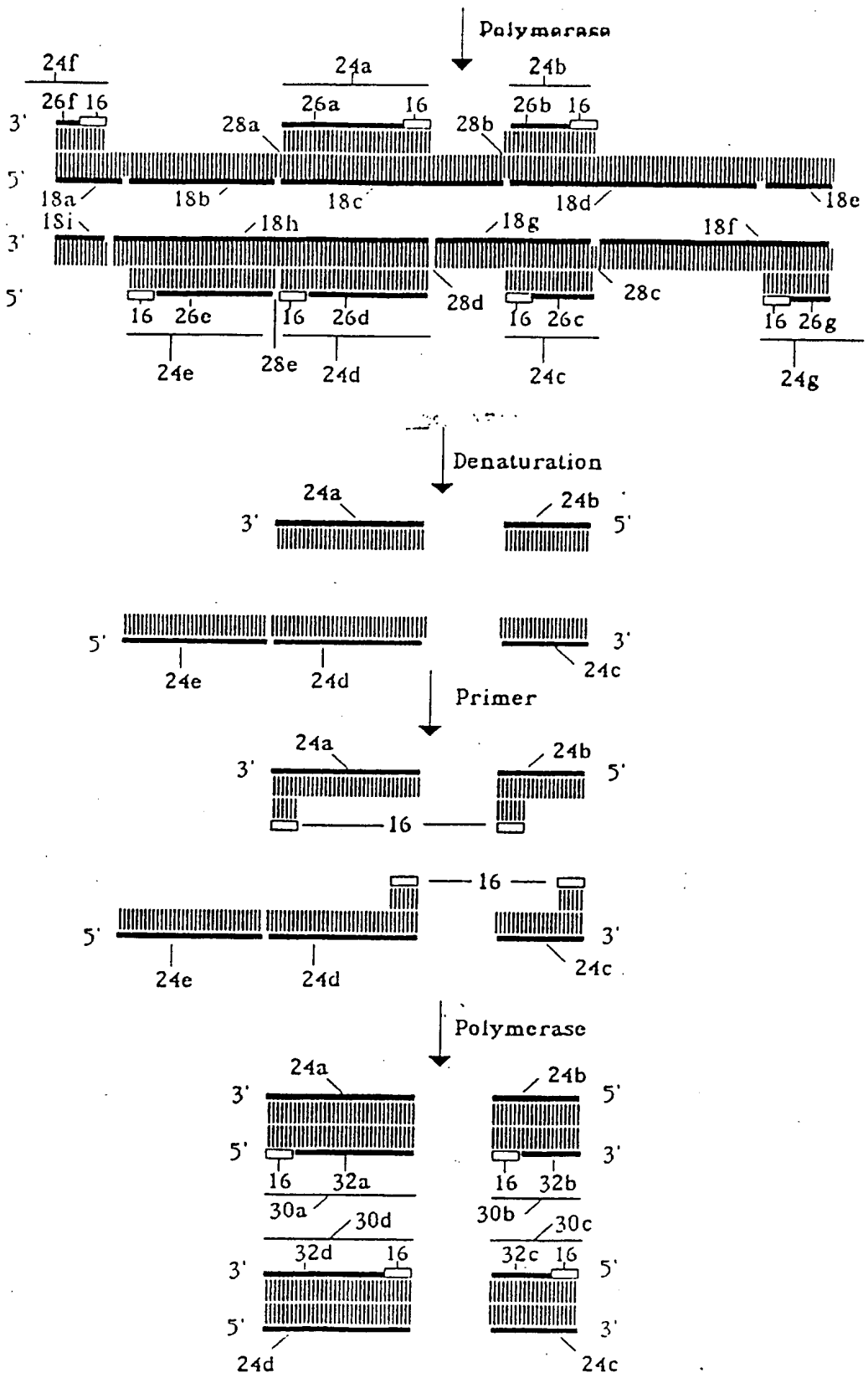

Fig 1 (continued)

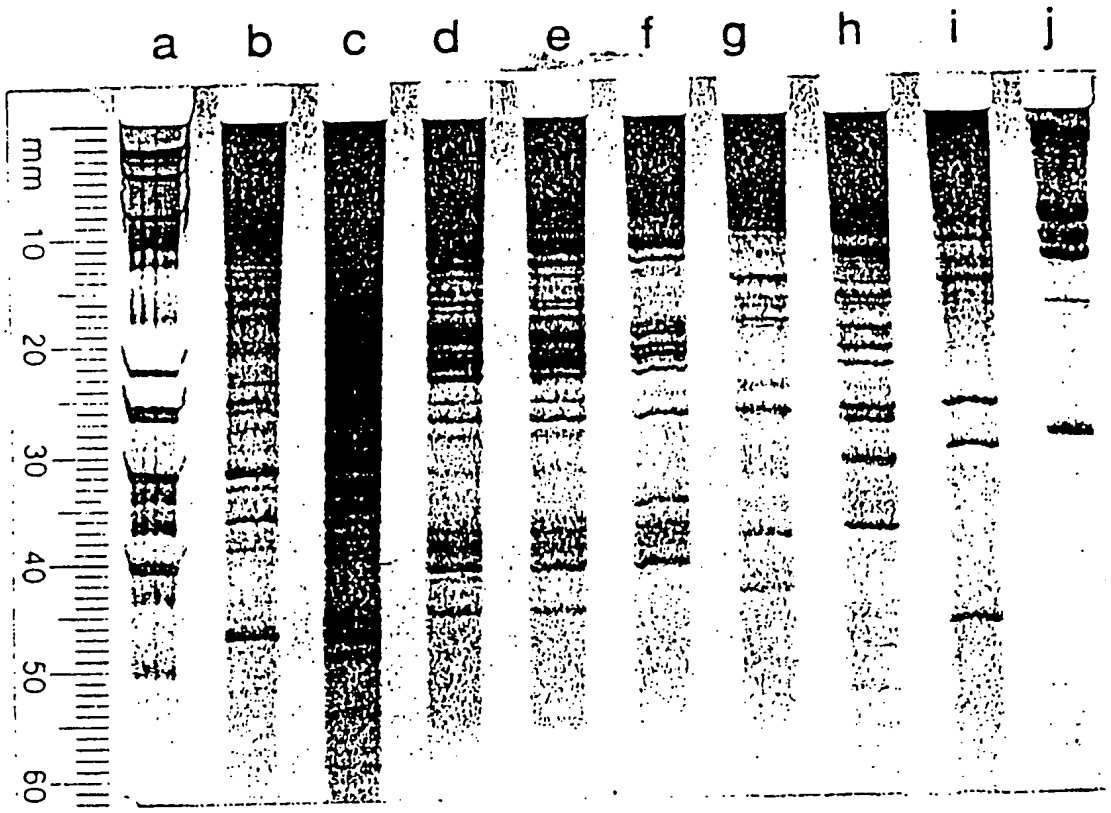

Fig. 2

Fig. 3

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06041

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12P 19/34
U.S. CL.: 435/91

II FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/6,91,172.3; 436/501; 536/27; 935/17.77,78,88

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US,A, 4,683,195 (MULLIS ET AL.) 28 July 1987, see entire disclosure, especially the abstract.	1,3-6
X	US,A,4,683,202 (MULLIS) 28 JULY 1987, see entire disclosure especially the abstract.	1,3-6
T	US,A, 5,043,272 (HARTLEY) 27 August 1991, see entire document especially the abstract.	1-5

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
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